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14. ABSTRACT

We propose that mammary stem cells could be involved in tumor development in either of two ways. The first is that altered function could change the course of development, and affect the susceptibility to transformation in adult life. We have found and characterized a model to test this hypothesis, and have preliminary array data to describe these cell populations. The second way that mammary stem cells could affect tumor development is by direct recruitment of these cells as tumor precursors. We have characterized a model of carcinogen-induced tumor development, and show that canonical stem cells are highly sensitive to genotoxins, and unlikely to be direct precursor cells. In order to study the factors that regulate mammary epithelial cell growth, we have chosen to examine their interaction in microchannels. These are very small-scale culture devices that allow for the culture of cells in defined media in low volumes. We have found that mammary stem/progenitor cells induce the division of the cell majority, suggesting an entirely novel function for this group of cells.

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mammary stem cells, modeling of aging, carcinogen-induced tranformation, microfluidic cultures

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C. M. Alexander

Introduction

We propose that somatic mammary stem cells could be involved in breast cancer in at least two ways. The first way is that their activity during development and maintenance of the gland could determine the growth potential, differentiation and senescence of the mammary epithelial cell population. This in turn will determine the response of mammary epithelial cells to carcinogenic challenge. The second is that they could serve as direct precursor cells for tumor development. Somatic stem cells have many of the properties associated with tumorigenic cells, with no need to invoke mutational changes (such as immortality and lack of dependence upon substrate adhesion). They may therefore represent a "fast-track" route to tumor development.

To evaluate whether stem cell activity determines the properties of the cell majority, we generated a mathematical model that described the fractions of mature and stem/progenitor cells in developing and adult mammary gland, that took into account well-understood biological aspects. Application of the model produced several clear and testable outcomes. We have begun to test these outcomes using a mouse carrying a null mutation in the Wnt signaling receptor, LRP5. Although there are relatively normal mammary ductal trees in these mice, they have an undetectable stem cell activity (Lindvall et al., 2006). This is therefore an ideal model to test our hypothesis that low stem cell activity will lead to premature aging and senescence of the mammary population.

Mice with a null mutation in syndecan-1 (Sdc1) resist tumor development in a number of different lineages (hematopoietic, breast, liver and lung) in response to Wnt oncogenes and carcinogen treatment. The reason for their resistance appears to lie in alterations during the initiation/recruitment phase of tumor development. These carcinogen-induced tumors appear to originate in a multipotential precursor cell. By studying these mice, we propose we can identify which process is susceptible to transformation during the response of stem/progenitor cells to carcinogen administration. Here, we report what happens to stem/progenitor cells after carcinogen exposure.

Clearly the mammary epithelial population is heterogeneous, and in vivo, various cell populations auto-regulate to manage the growth and differentiation of the mammary gland. In particular, stem and progenitor cells are required to be acutely responsive to major growth demands, but not to ongoing minor demands that are accommodated by more differentiated cells. There are no culture platforms to evaluate long and short-range growth regulators that are used by various mammary subpopulations to communicate to other cells. Using a microchannel format, we can show that different cell subpopulations have specific effects on other subpopulations. Specifically, we have found that ductal stem and progenitor cells can induce cell division in more mature cell fractions.

Results /Body

Aim 1

Demographic Modeling

LRP5 is a biomarker for mammary stem/progenitor cells. LRP5 is one of two lipoprotein-receptor related proteins that serve as Wnt signaling receptors (alongside Wnt ligand-binding Frizzled receptors; Fig 1). We have shown that LRP5 is expressed by a subset of parabasal cells in mouse mammary ducts, and that LRP5-/- mammary glands are resistant to Wnt1-induced tumor development (Lindvall et al., 2006). Adult LRP5-/- ductal trees grow out slowly, and the adult trees have negligible stem cell activity. This may be counter-intuitive to those avid believers in stem cells, but is predicted from the results of our math modeling. Modeling populations with little or no symmetric stem cell division, or with reduced or absent stem cells (that rely instead on the outgrowth and expansion of progenitors) shows that these populations grow slower, but are little

affected overall (normally there are ample reserves of growth potential set aside in the ductal tree). Additionally, we observe that LRP5-/- mammary epithelial cell (MEC) populations are depleted of basal cells (Fig 1B), which are associated with stem/progenitor cells, and find that LRP5-high cells co-purify with the MRU stem cell-enriched fraction (Fig.1C, D). These LRP5-high cells contain the majority of stem cell activity when tested for activity in vivo (Fig. 1E). We conclude that LRP5 is required for mammary stem cell activity, and is not expressed by the majority of MECs.

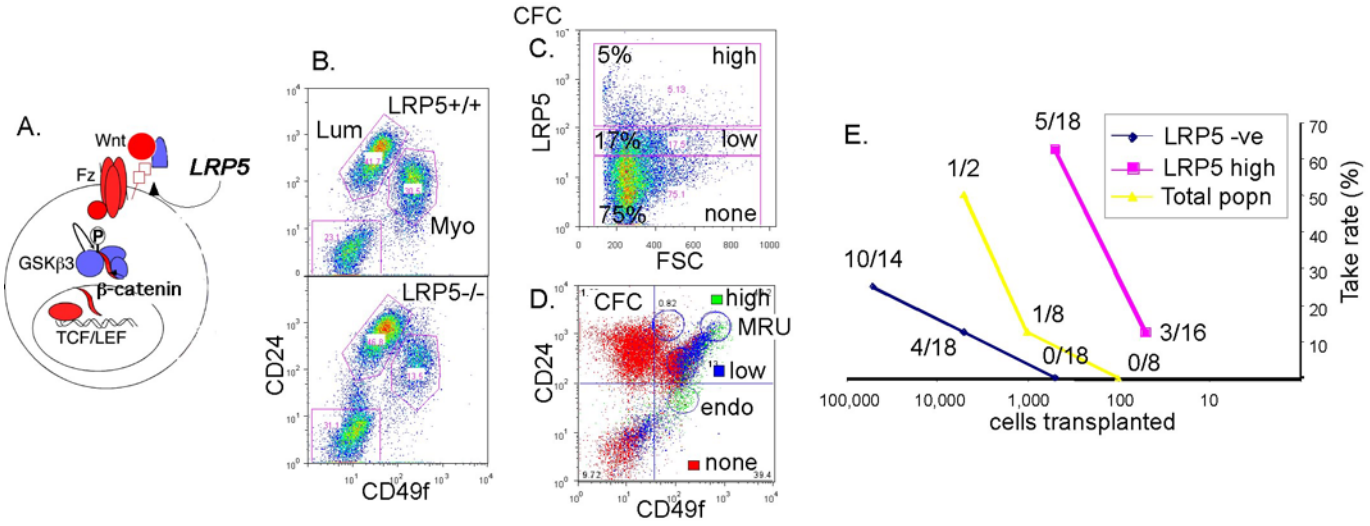


Fig. 1. LRP5 is a functional biomarker for mammary stem / progenitor cells. A. LRP5 is one of two signaling receptors for the canonical Wnt signaling pathway, which when activated recruits axin from the destruction complex and allows the signal transducer, β-catenin, to accumulate in the nucleus. B. MECs were analyzed by flow cytometry according to the protocol described by Stingl et al (Stingl et al., 2006). MECs were prepared from C57Bl6 and C57BL6: LRP5-/- virgin mice, and antibodies to CD24 and CD49f (α6 integrin) were added, along with antibodies to eliminate contaminating hematopoietic cells. This protocol will be called the "Stingl-gram". Myoepithelial cells (Myo) are separated from luminal cells (Lum) by this protocol (confirmed by us following separation and immunostaining of cytosplat preparations). In LRP5-/- MEC populations, the basal lineage (which contains the stem/progenitor fraction) is depleted, changing the luminal: myoepithelial ratio from 1.37 (control glands) to 3.54. **C.** MECs were incubated with anti-LRP5 antibody, and 3 fractions of high, low and undedectable levels of expression were superimposed upon the Stingl-gram (**D**). Two distinct stem/progenitor populations are labeled, clonagenic progenitors that grow in vitro (CFCs), and ductal stem cells that grow in vivo (MRU) and double negative (DN). The MRU fraction is highly enriched in LRP5-high cells. A distinct population that expresses high levels of LRP5 has been subsequently typed as von-Willebrand factor-positive endothelial cells (endo). **E**. LRP5-high and negative populations, together with a flow-sorted total population, were isolated and tested for their ability to colonize cleared fat pads. **The LRP5-high cells contain 99% of ductal stem cell activity.**

We propose that this LRP5-/- MEC population is ideal for testing our modeling outcomes, since without stem cells, there should be a substantial proportion of the MEC population that are hyper-differentiated or peri-senescent or more simply, aged. We have gathered array data from these mice, alongside mice that were naturally aged over 2 years, and will have the final, complete, multivariate data set in the next few months. With this in hand, we aim to develop biomarkers for aged cells, and progess to test their functional properties. Using this information, we propose we can anticipate the susceptibility of mammary glands to transformation.

Aim 2

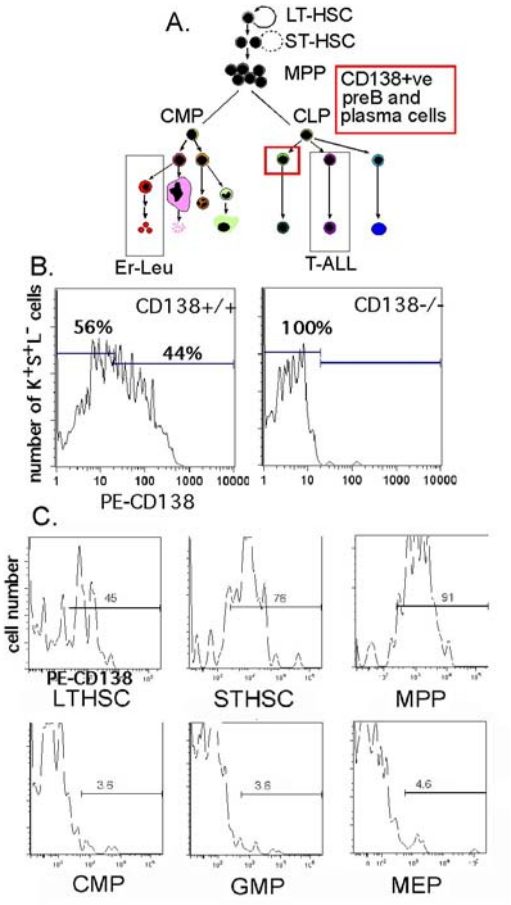
Carcinogen administration ablates mammary and hematopoietic stem cell compartments. Our data suggests that the Sdc1-/- allele may be effective at reducing tumorigenesis when stem/progenitor cells respond

to carcinogen administration. As a first step to finding out how Sdc1 potentiates tumor development, we have characterized the response of two types of somatic stem cell to carcinogen treatment. We use hematopoietic stem cells as a model for our studies in breast, since the isolation of HSCs and all subsequent cell compartments (progenitors, committed progenitors and mature cells) is so well characterized. In contrast, the properties and purity of fractions of mammary epithelial cells are not well understood.

Our work shows that there is a Sdc1-positive progenitor cell that emerges from the Sdc1-negative hematopoietic stem cell (Fig. 2). This is the most likely source of the hematopoietic malignancies that arise in several hematopoietic lineages. In the absence of Sdc1, the appearance of hematopoietic tumors is inhibited by 80%.

Fig. 2. Syndecan-1 (CD138) is expressed by cells in the hematopoietic stem/progenitor compartment. A. A scheme of hematopoietic differentiation that shows the lineages that resemble the diseases arising in carcinogen-treated mice (ErLeu and T-ALL), and the known expression site for CD138 (boxed in red). B. Bone marrow cells of control (CD138+/+) and Sdc1-/- (CD138-/-) mice were analyzed by flow cytometry, and 50% of a crude stem/-progenitor fraction (K+S+L-) was found to express CD138. C. Further purification of stem and progenitor fractions showed that CD138 was expressed by some LT-HSCs, most ST-HSCs,all MPPs (for further detail, see (McDermott et al., 2006)), but none of the more committed progenitors.

Similarly, the breast tumors that arise after carcinogen administration are bilineal (Fig. 3), and may have a bipotential precursor cell drawn from a stem/progenitor cell compartment.



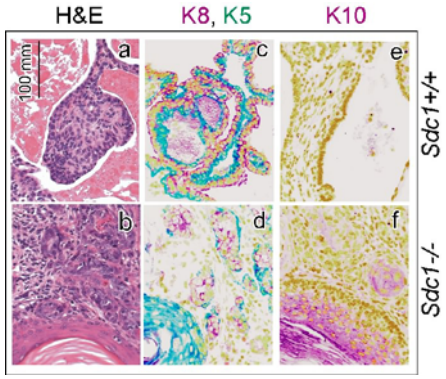


Fig. 3. DMBA administration to adults results in bi- or multi-lineal mammary tumors. Carcinogen-induced mammary tumors were evaluated by H&E staining, and for their expression of lineage markers for luminal cells (Keratin 8; K8) and myoepithelial cells (Keratin 5; K5) and for ectodermal / skin markers (Keratin 10; K10). Highly differentiated, cystic adenocarcinomas develop in Sdc1+/+ control strains (70% of total) and more aggressive acanthomas (including perlized squames and the expression of markers typical of skin) in Sdc1-/- mice (70% of total).

As a first attempt at defining the reaction of stem/progenitor cells to carcinogens, we have separated these fractions from mammary gland and from hematopoietic cells at various times after carcinogen administration. Female BALB/c mice were treated at 35 days when mammary ductal outgrowth was highest (Fig. 4 A), to ensure maximal stem cell activity. Though glands from these mice showed little gross effect 6 weeks later, analysis of the MECs by flow cytometry showed the same degree of basal cell depletion characteristic of LRP5-/- glands, and preliminary functional analysis suggests that this is associated with substantial stem cell ablation (Fig. 4 B, C). A similar analysis of hematopoietic stem /progenitor fractions also showed substantial ablation of stem cell fractions (97% within 3 days), though the overall bone marrow cellularity and circulating blood counts were not substantially affected (Fig. 4D). Thus, though the stem cell fractions appear to be specifically killed or arrested by carcinogens, the function of the lineage is perpetuated through other mechanisms.

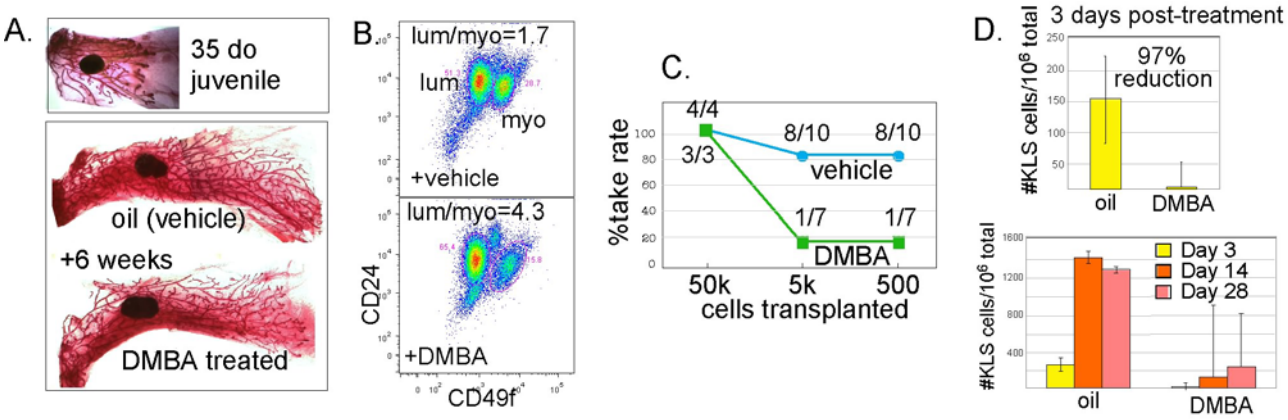


Fig. 4. Stem cells are ablated by DMBA administration. A. *BALB*/c mice were administered 0.1 ug/ml DMBA at 35 days of age, and allowed to develop for 6 weeks. Whole mount analysis of gross mammary morphology shows that DMBA administration does not affect development. **B**. MECs from DMBA and control mice were analyzed by flow cytometry, and found to show basal cell depletion. **C.** Preliminary functional analysis of MEC populations transferred to cleared fat pads suggests that there is substantial ablation of ductal stem cell activity. **D**. Hematopoietic stem/progenitor fractions (crude fractions isolated using Kit+Sca+ Lin- sorting) were isolated from mice administered DMBA as juveniles (12 days old). Three days after treatment, 97% of KSL cells were gone, though approximately normal blood counts were maintained. The recovery of this fraction was slow, only 18% after 4 weeks.

Aim 3

Interaction of mammary epithelial cell subpopulations

We propose that the various mammary subpopulations interact to balance the growth and differentiation of the gland. Using a microchannel culture vessel, we are able to study very low numbers of cells for their functional properties. Usually, it is impossible to keep such epithelial cells alive in low density macro-cultures, without complex feeder support and the addition of many undefined growth supplements. In the microchannels, we can do short-term cultures of very low cell numbers, with minimal addition of ectopic growth supplements. Using this platform, we can identify the normal regulators of mammary epithelial cell growth.

Our prior data suggested that there could be a rare cell type that was a particularly potent source of growth stimulation for the cell majority. To find out what this subpopulation is, we have purified different cell types from the total population, using flow cytometry. The growth potential of these fractions have been

defined by others (and confirmed by us). Thus, mammary stem cells are defined functionally as cells with the ability to reconstitute a mammary ductal tree in vivo (mammary repopulating units, MRUs, Fig. 5). Mammary progenitor cells are defined as having insignificant growth potential in vivo, but bipotential growth pattern in vitro (colony forming cells, CFCs). Other fractions include myoepithelial cells (MYO) and luminal cells (CD24lo).

We have recombined these flow-sorted fractions with "reporter cells" which comprise unsorted clusters of control cells. Our results are very interesting (Fig 5). They suggest that the stem and progenitor cell populations stimulate the growth of the majority. This implies that stem/progenitor cells not only serve as mothers for the cell majority, but also stimulate the growth of more differentiated cells further down the lineage.

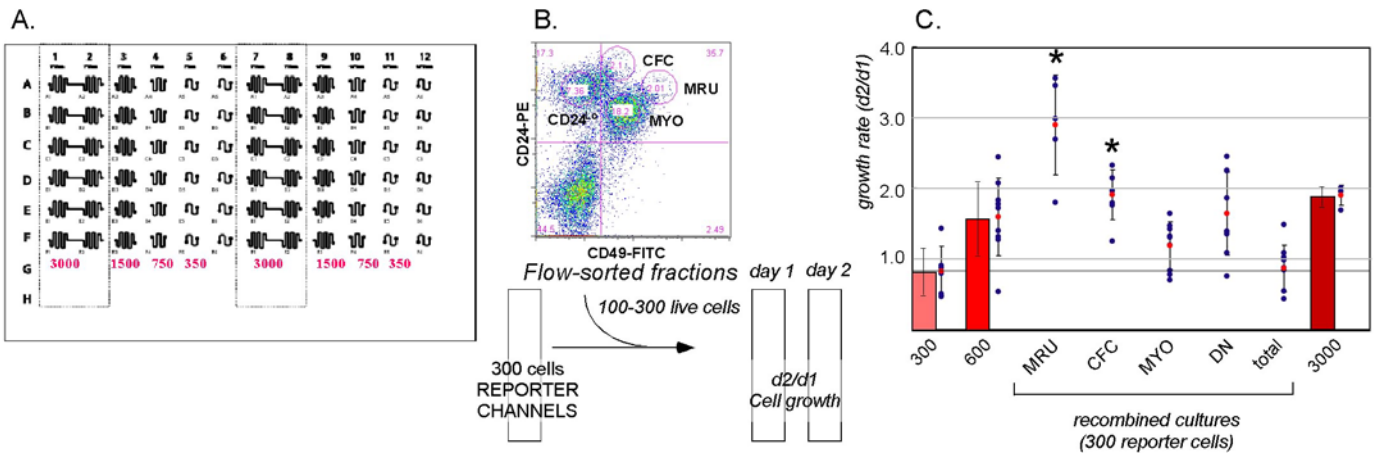


Fig. 5. Mammary stem/progenitor cells stimulate the growth of the cell majority. A. *Diagram of microchannel devices*. Microchannels of various length/volumes were seeded with cells to change only the total cell number seeded. This culture platform showed that the more cells were seeded, the more probable that rate of growth would be high. B. *Experimental Design*. Using the mid-size channels, recombinations of cells were tested for their growth rate. 300 reporter cells were recombined with flow sorted fractions enriched in stem cells (MRU), progenitors (CFCs), myoepithelial cell (MYO) or luminal cells (CD24lo). C. The growth rate of the resulting mixtures was measured (number of cells on day 2/number on day1). Control cultures that illustrate the normal range of growth observed for total populations were seeded in the devices of unequal length shown in (A). Thus different numbers of cells were seeded, to show d2/d1 for low (300) and high (3000) cell numbers (together with the high/low pattern shown for intermediate cell numbers (600). Statistically increased growth rates were observed when stem/ progenitor cell fractions were recombined with reporters.

Key Research Accomplishments

- Definition of a mammary stem cell biomarker
- Development of functional data to describe "aged" mammary epithelial populations
- Collection of transcriptional profiling data that describe the "aged" populations, with the aim of describing the signature set

- Description of cytotoxicity of carcinogens for stem cell populations (both mammary and hematopoietic)
- Evolution of the microfluidic platform for the evaluation of the functional properties of mammary epithelial cells

Reportable Outcomes

Patent filed:
Mammary Stem Cell Marker, Alexander et al P07136US

Degrees obtained:
Hongmei Yu, PhD, Biomedical Engineering,

Conclusion

We have developed a model for evaluating the effect of depletion of normal stem cell numbers on mammary development, and this will serve to test the predictions of our mathematical model. We will be able to evaluate the properties of the adult cell majority under these circumstances. We have shown that stem cells are very sensitive to the effects of genotoxins. The implication of this observation is that if there is recruitment of an undifferentiated cell precursor during tumorigenesis, it will be from the progenitor cell compartment. The microchannel culture platform has shown that stem / progenitor cells have growth-promoting activity for the cell majority, and this suggests an unorthodox activity of stem cells during tissue regeneration.

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